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Evidence of vertical transmission and tissue tropism of Streptococcosis from naturally infected red tilapia (*Oreochromis* spp.)

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ABSTRACT

Streptococcosis is a highly problematic disease in the aquaculture of freshwater fishes, especially for tilapia. The possibility of vertical transmission of streptococcosis and the pattern of tissue tropism of this pathogen in various organs was examined in red tilapia (*Oreochromis* sp.). Healthy broodstock without any clinical signs of *Streptococcus* spp. were selected from a farm earlier reported to have the disease and a total of 10 pairs were forced spawned to provide samples of gametes and progeny for pathogen testing. A colorimetric LAMP assay was used to confirm whether the bacterial pathogens *Streptococcus. agalactiae* and *Streptococcus. iniae* was present in samples of milt, unfertilized eggs, fertilized eggs, and offspring at various stages of development, as well as internal organs of broodstock (reproductive organs, gill, liver, spleen, kidney and brain) as well as samples of water from culture systems. The majority of samples of milt (9/10) and unfertilized eggs (7/10) collected from the broodstock were infected with *S. iniae* at the time of spawning and was transmitted to all of their offspring. Nevertheless, when the same samples of gametes were analyzed for *S. agalactiae*, they were all found to be negative but the pathogen was found to be present in some 10-day-old larval offspring (4/10). However, when the pathogenic presence was analyzed from the reproductive organs of the parents, both *S. agalactiae* (11/20) and *S. iniae* (18/20) bacterium were common. Although, all broodstock were asymptomatic, almost all broodstock harboured the bacteria in many organs. Confirmation of vertical transmission of streptococcosis in tilapia means that intergenerational break cannot be used as a reliable and simple means of reducing or eliminating the prevalence of these difficult pathogens in aquaculture stock.

1. Introduction

Streptococcosis is a disease resulting from infection by members of a diverse group of bacteria (*Streptococcus* spp. and *Lactococcus* sp.) that possess the capacity to infect a wide range of hosts, including humans (Johri et al., 2006; Amal and Zamri-Saad, 2011; Plumb and Hanson, 2011). Streptococcosis outbreaks in tilapia have been reported from many parts of the world (Al-Harbi, 1994; Abuseliana et al., 2010; Eldar et al., 1994; Perera et al., 1994; Shoemaker et al., 2001) with the disease becoming a major problem in tilapia aquaculture with total global losses in production in 2008 estimated at US$250 M (Klesius et al., 2008; Shoemaker and Klesius, 1997). High mortality rates of more than 50–70% over a period of 3–7 days are common (Wongsathein, 2012; Yanong and Francis-Floyd, 2002)

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that are frequently associated with intensive aquaculture systems (Hernandez et al., 2009; Shoemaker et al., 2001; Salvador et al., 2005). The most significant causative bacteria of streptococcosis in fish worldwide includes; *S. iniae*, *S. agalactiae, S. parauberis, S. difficile*, *S. shiloi* and *S. dysgalactiae* (Abuseliana et al., 2010; Costa et al., 2014; Eldar et al., 1994; Evans et al., 2006; Figueiredo et al., 2012; Haines et al., 2013; Mata et al., 2004; Maisak et al., 2008; Netto et al., 2011; Zhang et al., 2003). In Thailand, where tilapia aquaculture is extensive, both *S. agalactiae* and *S. iniae* have been reported as the most prevalent causative species (Rodkhum et al., 2012; Suanyuk et al., 2008, 2010), however, they are difficult to distinguish due to their similar microbiological appearance and clinical signs. According to their biochemical characterization and properties, *S. agalactiae* belongs to Lancefield group B Streptococcus, Gram-positive cocci, with either β-or non-haemolytic (γ), non-motile, oxidase negative properties (Wongsathein, 2012), while, *S. iniae* is a non-Lancefield, Gram-positive coccus bacteria with catalase-and oxidase negative, non-motile and β-haemolytic properties (Agnew and Barnes, 2007). Clinical signs and symptoms of streptococcosis in fish may vary depending on the species of fish, however, the most common symptoms include loss of orientation and erratic swimming, unilateral or bilateral exophthalmia, anorexia, eye opacity, abdominal distention, darkening of skin and hemorrhaging skin around the anus or at the base of the fins (Amal and Zamri-Saad, 2011; Amal et al., 2013a; Evans et al., 2002; Karsidani et al., 2010).

The transmission of *Streptococcus* spp. in tilapia via a range of experimental routes has been demonstrated, including intraperitoneal injections, immersion, cohabitation with infected fish, as well as oral, gill and nare inoculation (Evans et al., 2000; Perera et al., 1994; Shoemaker et al., 2000). Under natural conditions the main pathways of disease transmission appears to be through direct contact between healthy fish and diseased or dead fish, as well as indirect contact via the water in culture systems (Agnew and Barnes, 2007,b; Amal et al., 2013a,b; Bowater et al., 2012; Kim et al., 2007; Nguyen et al., 2002; Robinson and Meyer, 1966). A number of studies confirm the horizontal transmission of *Streptococcus* spp. in tilapia (Amal et al., 2013b; Evans et al., 2000; Hernandez et al., 2009; Hossain et al., 2014; Jimenez et al., 2007; Mian et al., 2009; Shoemaker et al., 2000), whereas natural outbreaks of the disease on tilapia farms indicate that larvae and juvenile fish smaller than 20 g may not be susceptible (Hernandez et al., 2009; Jimenez et al., 2007), suggesting that little or no vertical transmission of the disease.

A recent study detected both *S. agalactiae* and *S. iniae* in the reproductive organs (testis and ovary) as well as in the fertilized eggs and fingerlings derived from apparently healthy broodstock of hybrids of Nile and red tilapia (Suebsing et al., 2013). These results suggest that vertical transmission of these bacterial diseases to progeny may also be possible in tilapia. To test this proposition, gametes were sampled from individual broodstock under quarantine conditions and used for *in vitro* fertilization and subsequent culture of offspring. Major organs of the broodstock as well as samples of milt, unfertilized eggs, fertilized eggs and progeny at various ages were screened for the presence of both *S. agalactiae* and *S. iniae* to determine the possible vertical transmission of these pathogens.

2. Materials and methods

2.1. Broodstock source and maintenance

A total of 40 female and 20 male adult red tilapia (*Oreochromis* sp.) of 150–200 g in size were randomly selected from a commercial farm stock held at Naam Sai Farm, in Prachinburi Province, in eastern Thailand. All of the adult fish were apparently healthy and had previously been routinely used as broodstock for the commercial production of fingerlings. They were then transferred in water to quarantine aquaculture research facilities where they were maintained in aerated 2 t round polyethylene tanks at a stocking density 12 m^2 with a sex ratio (male:female) of 1:3. The fish were conditioned for a month by feeding with standard commercial floating tilapia feed pellets (CP feeds) containing 28% crude protein at a rate of 4% of their biomass three times a day. In addition to the pelleted feed, fishes were also given duckweed (*Lemna* spp.) ad-libitum once a week as a supplementary natural feed (Pradeep et al., 2012). Water quality was maintained to ensure optimum conditions for the fish through adequate water exchange in the tanks salinity 0 ppt; total ammonia nitrogen <0.5 ppm; total nitrite <0.5 ppm; dissolved oxygen >5 ppm; pH 7.0–8.0. Filtered (1 μm) and UV sterilized bore water was used to minimize the potential for introduction of water-borne pathogens. Broodstock and larval rearing tanks for the experiment were strictly maintained under quarantine conditions in an isolated fish rearing experimental unit to reduce problems with internal contamination, where all the handling equipment, floors and tanks were routinely disinfected using iodine povidone as a bio-security measure.

2.2. Artificial fertilization

Synchronized breeding, gamete collection and artificial fertilization from the tilapia broodstock were followed according to protocols described previously (Pradeep et al., 2010) and were conducted under the quarantine conditions. The broodstock were starved for 24 h and the females were then inspected individually to determine the stage of maturity and only those individuals ready to spawn ($n = 10$) were selected for subsequent stripping of eggs into a dry sterilized Petri dish. For each female about 20 unfertilized eggs were then sub-sampled and transferred to a microcentrifuge tube (1.5 ml) for pathogen screening. A matching number of male fish ($n = 10$) were also selected and stripped of milt after blotting the fish dry to avoid any possible activation or contamination of the sperm, while withdrawing the milt directly into fine capillary tubes (6 tubes each of 50 μl) whilst taking extreme care to avoid any contamination with urine. Three capillary tubes of sperm were transferred to a microcentrifuge tube (1.5 ml) for pathogen screening. The remaining three capillary tubes of sperm from a male fish were then used to fertilize eggs from a female fish by spreading the sperm over the eggs placed in the Petri dish. To activate the sperm for artificially inseminating the eggs, 15 ml of UV sterilized fresh water (28 ± 1 °C) was added to the petri dish and was shaken gently for thorough mixing of the sperm with eggs. After two minutes, excess water was decanted from the petri dish and an additional 15 ml of UV sterilized fresh water (28 ± 1 °C) was added and then drained. After approximately 1 h 20 fertilized eggs were sub-sampled into a 1.5 ml microcentrifuge tube for subsequent pathogen screening, and the remaining eggs were carefully moved into a round-bottomed hatching chamber provided with UV sterilized water (Pradeep et al., 2011). Upon hatching, the larvae were moved from the hatching chamber to an individual larval rearing tank (100 l), which was maintained with filtered (1 μm) UV sterilized water and reared until the termination of the experiment after 30 days from hatch. The water quality in the larval culture tanks was closely monitored and any dead larvae or debris were removed at regular intervals. The larvae were feed with powdered feed (CP feeds) (28% protein) at a ration of 12–15% of their body weight three times a day for a period of 10 days and then the feeding ration was subsequently reduced to 8–10% of the total biomass.

2.3. Sample collection

A total of 10 pairs of tilapia broodstock were artificially bred and samples of gametes and larvae collected for testing for the